ORIGINAL ARTICLE

Salbutamol exhibits androgenic activity in vitro

André O von Bueren, Risheng Ma, Margret Schlumpf, Walter Lichtensteiger

Br J Sports Med 2007;**41**:874–878. doi: 10.1136/bjsm.2007.035162

See end of article for authors' affiliations

Correspondence to: André O von Bueren, University Children's Hospital, Steinwiesstrasse 75, CH-8032 Zurich, Switzerland; Andre.vonBueren@kispi.uzh.ch

Accepted 5 May 2007
Published Online First 17 May 2007

Background: Salbutamol has been shown to mediate anabolic effects after intravenous administration. However, the mechanism responsible for the anabolic actions of salbutamol remains unknown.
Aim: To investigate the potential mechanism by which salbutamol mediates anabolic effects in vitro.
Methods: The potential androgenic activity of salbutamol was investigated in vitro by the A-Screen assay that measures androgen-dependent inhibition of proliferation of the androgen receptor (AR)-positive human mammary carcinoma cell line, MCF7-AR1.
Results: The assay was validated with three known androgens; methyltrienolone (R1881), 5α-dihydrotestosterone (DHT) and danazol. IC_{50} values of R1881, DHT and danazol, 4.41×10^{-11}, 4.44×10^{-11} and 1.08×10^{-8} M, respectively, were in the ranges known from earlier studies. Our results demonstrate that salbutamol exhibits androgenic activity, with an IC_{50} value of 8.93×10^{-6} M. Anti-estrogenic or cytotoxic effects, which might have interfered with the assay, were excluded by additional experiments on wild-type MCF7 and MCF7-AR1 cells, respectively.
Conclusion: These data indicate that salbutamol exerts anabolic effects through androgen receptor agonistic activity in vitro.

Asthma, a chronic inflammatory airway disorder, is one of the most frequently occurring chronic diseases. Its prevalence in adults is about 5%. Among athletes, however, it is assumed to be 10–20%.[1] Inhaled beta-2 agonists are the drug of choice for treatment of asthma. However, beta-2 agonists are prohibited for non-asthmatic athletes according to the most recent list of prohibited substances released by the World Anti-Doping Agency (WADA).[2] The main reason for prohibition in non-asthmatic athletes is its claimed ergogenic potential.

Beta-2 agonists have received recent attention from the International Olympic Committee (IOC) and the World Anti-Doping Agency. At the 1992 Barcelona Olympic Games, two US athletes tested positive for the beta-2 agonist clenbuterol and were subsequently disqualified. Controversy at the Olympics about clenbuterol raised questions as to whether beta-2 agonists could affect muscle mass and function.[3–6] Numerous studies have demonstrated that administration of some beta-2 agonists such as clenbuterol to a variety of species caused muscle growth and alteration in body composition.[7 8] However, the anabolic effects of salbutamol remain equivocal. In animal studies, the anabolic effect of salbutamol on skeletal muscle was only found after intravenous administration with implanted minipumps,[22] but never after oral administration.[11 21] Oral administration of slow-release preparation of salbutamol (Volmax, Glaxo, Greenford, UK), caused increases in quadricep and hamstring muscle strength, but did not affect lean body mass in healthy men.[9]

A key question regarding the biochemical basis of potential salbutamol-mediated anabolic effects concerns whether salbutamol activates such effects through beta-adrenoreceptor stimulation or by other mechanisms. Beta-2 agonist-mediated anabolic effects on muscle have been reported to be dependent and independent of actions on beta-adrenoreceptors.[10–12]

We tested salbutamol for possible actions on androgen and oestrogen receptors in vitro. A possible androgenic action of salbutamol was investigated by the A-Screen assay, which measures androgen-dependent inhibition of proliferation of the androgen receptor (AR)-positive human mammary carcinoma cell line, MCF7-AR1. This cell line has been stably transfected with a full human AR;[13] it expresses approximately five times more AR than wild-type MCF7 cells. MCF7-AR1 cells retain the capacity to proliferate in response to oestrogen treatment. Androgens inhibit oestrogen-induced proliferation and cells arrest in G0/G1 phase.[13 14] We validated the assay with known synthetic and natural androgens, and tested the activity of salbutamol.

METHODS

Cell lines and culture conditions

MCF7 human breast cancer cells and MCF7-AR1 cells were kindly donated by Ana Soto and Carlos Sonnenschein, Tufts University, Boston, Massachusetts, USA. MCF7 cells at passage 10, MCF7-AR1 cells at passage 18. The MCF7-AR1 cell line has been stably transfected with a full human AR.[13] MCF7 and MCF7-AR1 cells were grown in Dulbecco's modified Eagle's Medium (DMEM) with phenol red (GIBCO, Grand Island, New York, USA) supplemented with 5% heat inactivated (56°C, 30 min) foetal bovine serum (FBS) (GIBCO) and 2 nM L-glutamine (GIBCO). This medium was used as growth medium to keep the cells in culture. Phenol red is commonly used in media as a pH-indicator, but is also a weak oestrogen.[15] Cells become proliferatively quiescent when transferred into phenol red-free DMEM supplemented with 10% charcoal–dextran treated foetal bovine serum (CD-FBS, steroid free), 2 nM L-glutamine, 10 mM Hepes buffer 1 M (GIBCO). This medium was used as the experimental medium.

Chemicals

17β-Estradiol was purchased from Calbiochem (Richmond, Virginia, USA), methyltrienolone (R1881) was from NEN Life Science Products (Boston, Massachusetts, USA), 5α-dihydrotestosterone (DHT) and salbutamol was from Fluka (Buchs,

Abbreviations: AR, androgen receptor; CD-FBS, charcoal–dextran treated foetal bovine serum; DHT, 5α-dihydrotestosterone; OD, optical density; SRB, sulforhodamine B; TCA, trichloroacetic acid

Switzerland) and danazol was from Sigma-Aldrich Chemie (Schnelldorf, Germany).

Estrogenic and anti-estrogenic activity

The E-Screen assay was conducted as described in recent studies.[16 17] Briefly, on the first day, MCF7 cells were trypsinised and plated into Costar 96-well plates at initial concentration of 3000 cells per well in 100 µl experimental medium, and allowed to attach. On the second day 100 µl experimental medium containing the various dilutions of test chemicals was added into each well. Cells were incubated for a total of 6 days. The assay compares the cell number of MCF7 cells in the absence of any estrogens (negative control), in the presence of 10^{-10} M 17β-estradiol (positive control) and in the presence of a range of test chemical concentrations. Anti-estrogenic activity was tested by comparing the proliferative effect of 10^{-10} M 17β-estradiol with the proliferation induced by 10^{-10} M 17β-estradiol in the presence of increasing concentrations of test chemicals. Each concentration was tested in six wells.

Androgenic activity

The A-Screen assay compares the cell number of MCF7-AR1 cells in the absence of any estrogens and androgens (negative control), in the presence of 10^{-10} M 17β-estradiol (oestrogen control), and in the presence of 10^{-10} M 17β-estradiol together with a range of test chemical concentrations.[14] On the first day, MCF7-AR1 cells were trypsinised and plated into Costar 96-well plates at initial concentration of 5000 cells per well in 100 µl experimental medium, and allowed to attach. On the second day, 100 µl experimental medium containing the various dilutions of test chemicals was added into each well. Each concentration was tested in six wells. Negative control (absence of any estrogens and androgens) and positive control (10^{-10} M 17β-estradiol) were also tested in six wells. In addition, an androgen response curve was set up as positive control in every experiment. Cells were incubated for 5 days.

Fixation protocol and sulforhodamine B (SRB) colourimetric assay

A- Screen and E-Screen assays were terminated by aspirating the media from the wells. Cell number was assessed by measurement of total protein content using the SRB assay.[18 19] Within a certain range of SRB concentrations, the extinction of SRB at 492 nm is directly proportional to the cell number. The experimental medium was gently discarded, then fixed for 30 min with 100 µl cold 4℃ 10% trichloroacetic acid (TCA) (Merck, Whitehouse Station, New Jersey, USA). Plates were left for 30 min at 4℃ and subsequently washed 5 times with deionised water. Plates were then left to dry at room temperature for at least 24 h. Cells were stained by adding 100 µl 0.4% SRB (Sigma Chemical Co, St Louis, Missouri, USA) in 1% acetic acid to each well for 15–20 min at room temperature, protected from light, in order to avoid fading of the colour. The SRB was then removed and the plates were washed 5 times with 1% acetic acid before air-drying. Bound SRB was solubilised with 100 µl 10 mM Tris buffer pH 10.6 (unbuffered Tris-base solution) and plates were left on a plate shaker for at least 10 min. Absorbance was read in a 96-well plate reader (Anthos Labtec 2001, Anthos Labtec Instruments, Wals, Austria) at 492 nm, subtracting the background measurement at 620 nm. The optical density (OD) test value was defined as the absorbance of each individual well. To establish a linear relationship between OD and cell number, defined numbers of cells between 0 and 9×10^5 per well were seeded into 96-well plates. After an attaching time of 30 h, the cells were fixed and stained as described above (fig 1).

Cytotoxicity

Cytotoxicity was tested on MCF7-AR1 cells. Cells were trypsinised and plated into Costar 24-well plates at initial concentration of 2.5×10^4 cells per well in 1 ml growth medium, and allowed to attach. After 24 h the growth medium was aspirated and replaced with 0.5 ml experimental medium alone, with experimental medium in presence of 10^{-10} M 17β-estradiol, and with 0.5 ml experimental medium in presence of 10^{-10} M 17β-estradiol with a range of test chemical concentrations. After incubation for 5 days, the experimental medium was aspirated and the cells were trypsinised. Viable cells were counted according to the manufacturer's protocol. A total of 0.5 ml of a suitable cell suspension was placed into a tube and 0.1 ml of 0.4% Trypan Blue Stain was added. After thorough mixing, the solution was allowed to stand 5 min at room temperature. Cells were counted in a haematometer. This method stains non-viable cells.

Statistical analysis

The results were expressed as the mean and standard deviation (SD) determined in Microsoft Excel 2000 (Microsoft, Redmond, Washington, USA). Dose-response curves and IC_{50} values were calculated with Graphpad Prism V.2.01 for Windows (Graphpad, San Diego, California). Data were analysed by ANOVA followed by Bonferroni pairwise comparison.

RESULTS

Validation of the A-Screen assay

In order to evaluate the A-Screen assay we tested three androgens. The full agonists, R1881 and DHT, and the partial agonist, danazol, inhibited the proliferation of MCF7-AR1 cells, induced by 10^{-10} M 17β-estradiol, in a dose-dependent manner (fig 2A–C). R1881 and DHT were equally potent. Danazol was less potent. IC_{50} values (concentration required for 50% of maximal inhibition of 17β-estradiol-induced proliferation) of R1881, DHT and danazol, 4.41×10^{-11}, 4.44×10^{-11} and 1.08×10^{-8} M, were comparable with earlier studies.[13 16]

Effect of salbutamol

Salbutamol decreased 17β-estradiol-induced proliferation of MCF7-AR1 cells in a concentration-dependent manner (fig 2D), similar to the known androgens, with an IC_{50} value of 8.93×10^{-6} M. In order to answer the question of whether this inhibitory effect might be due to an AR agonistic action or to an anti-estrogenic effect, we added salbutamol to MCF7 cells in presence of 10^{-10} M 17β-estradiol. Salbutamol did not exhibit anti-estrogenic activity (fig 3).

Possible cytotoxic actions of salbutamol were assessed by incubating MCF7-AR1 cells for 6 days with 10^{-10} M 17β-estradiol alone, and by incubating MCF7-AR1 cells for 6 days in the presence of 10^{-10} M 17β-estradiol with high concentrations of salbutamol (3×10^{-6} M, 1×10^{-6} M, 3×10^{-5} M, 6×10^{-5} M). As possible androgenic actions of salbutamol on MCF7-AR1 cells were tested in the presence of 17β-estradiol, cytotoxicity was also assessed in the presence of 17β-estradiol. This experiment showed 84% viable cells when MCF7-AR1 cells were treated with 10^{-10} M 17β-estradiol alone. MCF7-AR1 cells treated with 10^{-10} M 17β-estradiol and the four different concentrations of salbutamol exhibited 86%, 92%, 83% and 84% of viable cells. Thus, the addition of salbutamol did not change the number of viable cells at the concentrations tested.

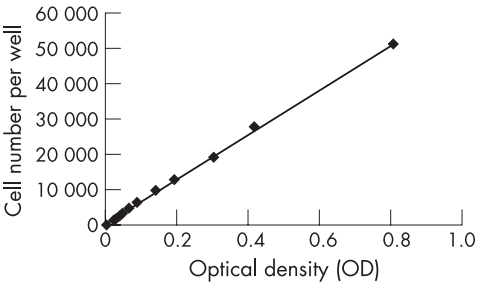

Figure 1 Linear correlation between number of cells per well and optical density: y (cells) = 63.161 × (extinction) +90.053; $R^2 = 0.9991$.

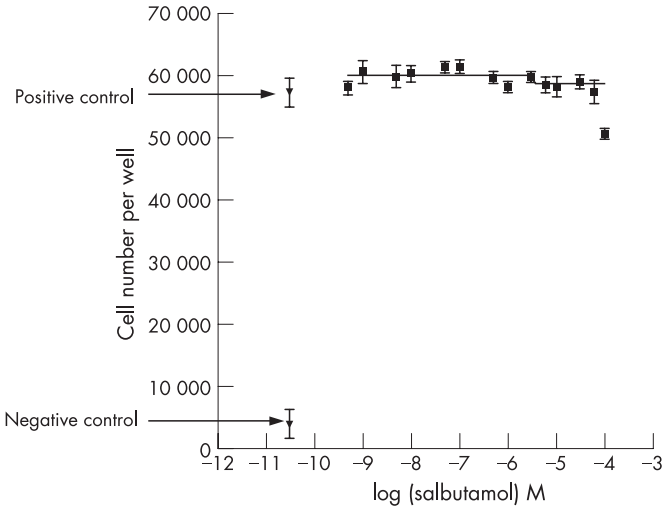

Figure 3 Proliferation of MCF7 cells in response to 17β-estradiol mixed with different concentrations of salbutamol. Mean (SD). Negative control was experimental medium only. Positive control was 10^{-10} M 17β-estradiol alone. Data were from two independent experiments.

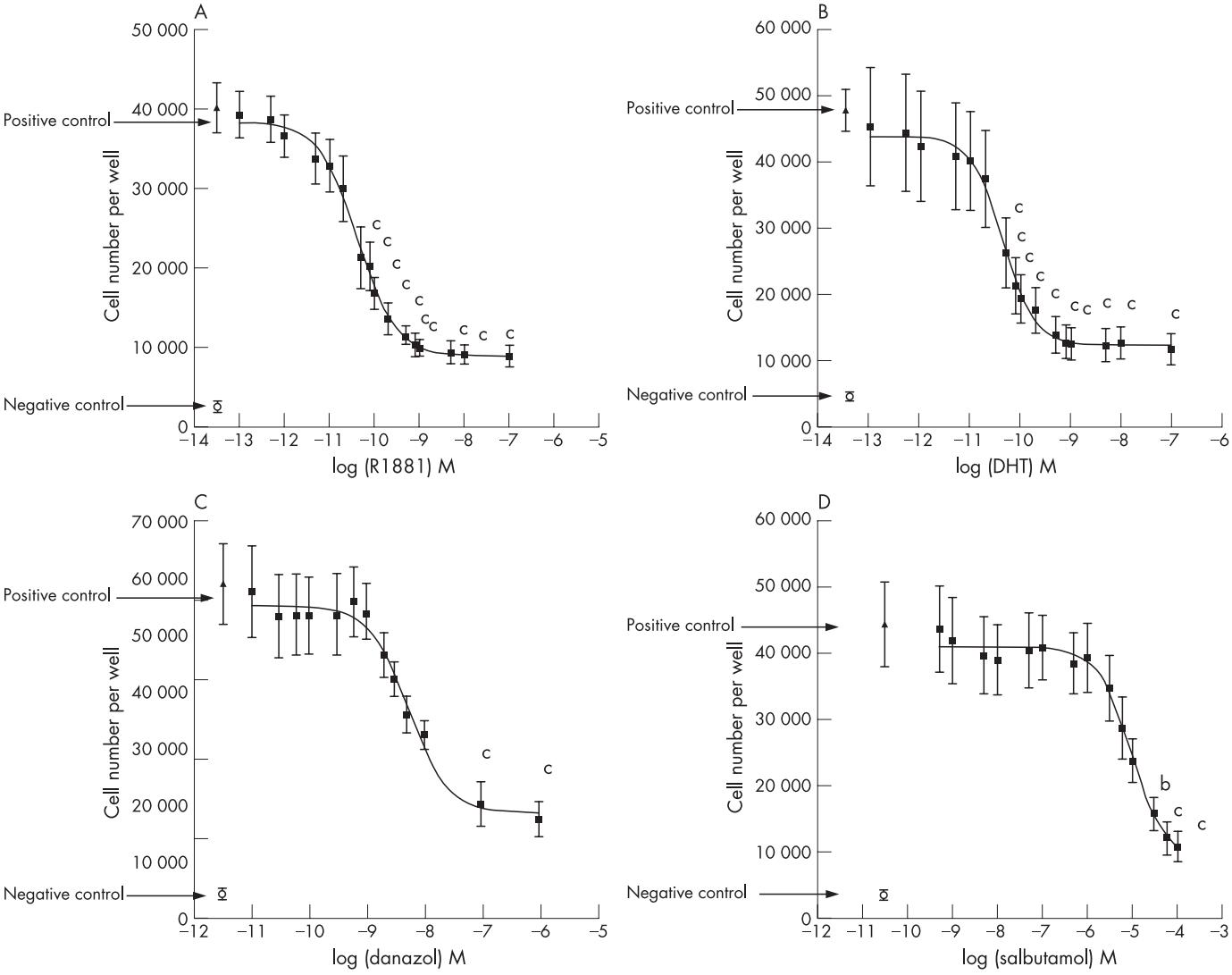

Figure 2 Inhibition of 17β-estradiol-induced proliferation of MCF7-AR1 cells by methyltrienolone (R1881) (A), 5α-dihydrotestosterone (DHT) (B), danazol (C) and salbutamol (D). Mean (SD). (A: n = 5; B: n = 5; C: n = 8, D: n = 5). Negative control was experimental medium only. Positive control was 10^{-10} M 17β-estradiol alone. Significant difference from positive control: B, $p<0.01$; C, $p<0.001$.

What is already known on this topic

- In animal studies, anabolic effects of salbutamol on skeletal muscle have been reported following intravenous administration.
- The mechanism underlying the salbutamol-mediated anabolic effects has yet to be investigated.

What this study adds

- Salbutamol indeed possesses androgenic activity.
- Novel biological insights about the potential mechanism of salbutamol-mediated anabolic effects are elucidated.

DISCUSSION

In the present study we used a biological assay to detect the androgenic activity of test chemicals. Proliferation of MCF7-AR1 cells, grown in the presence of 10^{-10} M 17β-estradiol was inhibited by all three androgens tested, in a concentration-dependent manner. The AR agonists R1881, DHT and danazol displayed IC_{50} values corresponding to the range reported in earlier studies.[13 16] This indicates that the cell system is able to detect full and partial AR agonists at low concentrations. The stable expression of a full-length, active androgen receptor in MCF7-AR1 cells did not appear to modify their proliferative response to estrogens when compared with the response of parental MCF7 cells.

Salbutamol also inhibited 17β-estradiol-induced proliferation of MCF7-AR1 cells. To elucidate whether this reduction of proliferation was due to cytotoxic, anti-estrogenic or androgenic effects, we carried out further experiments. Cytotoxicity was excluded in an experimental set-up analogous to that used for analysis of androgenic activity, by counting viable cells after incubation with 17β-estradiol in the presence or absence of salbutamol. As there was no difference in viability of cells treated with 17β-estradiol in the presence or absence of salbutamol, the inhibition of 17β-estradiol-induced proliferation of MCF7-AR1 cells by salbutamol does not appear to result from cytotoxic actions.

An anti-estrogenic effect can also be excluded, because salbutamol added to (wild-type) MCF7 cells in the presence of 10^{-10} M 17β-estradiol did not change cell proliferation (fig 3).

We conclude from the absence of anti-estrogenic or cytotoxic effects of salbutamol at the concentrations tested, that the inhibition of oestrogen-induced MCF7-AR1 cell proliferation by salbutamol is due to an androgenic action. However, further studies are needed to elucidate whether salbutamol is androgenic in vivo.

Our results add to the growing body of evidence that salbutamol mediates anabolic effects, and we report a mechanism by which salbutamol might promote such effects. Salbutamol has a short plasma half-life (3–6 h).[4] Following a recommended dose of inhaled salbutamol, the compound is only transiently detectable in the plasma,[4] and salbutamol reaches levels up to 2.5 ng/ml or 1.04×10^{-8} M.[27] These levels are considerably lower than effective concentrations in our in vitro experiments, which were above 10^{-6} M with an (IC_{50} value of 8.93×10^{-6} M). Whereas anabolic properties have been demonstrated in several species after systemic use of some beta-2 agonists,[11 20 21] salbutamol possess anabolic activity only after intravenous administration,[22] and no potent anabolic effects have been reported after oral administration in animals.[11 21] In addition, in humans, oral administration of therapeutic levels of salbutamol increases maximal anaerobic power,[24–26] irrespective of the subjects' training status, without changing the body composition.[25 26] Similar improvements in performance have been shown in two other studies, using a comparable daily salbutamol dose.[9 23] Of note, there is no evidence that inhaled beta-2 agonists have ergogenic effects at therapeutic doses.[28] Although it is too early to draw final conclusions, based on these studies we hypothesise that the agonistic activity we observed at the androgen receptor might play also a role after intravenous administration of salbutamol.

In summary, our results indicate that salbutamol indeed possesses androgenic activity, and provides new insights into salbutamol-mediated anabolic effects in vitro. Whether salbutamol is also an AR agonist in vivo remains to be clarified.

ACKNOWLEDGEMENTS

This project was supported by the Swiss National Research Program NRP 50. MCF7-AR1 and MCF7 cells were a gift of Ana Soto and Carlos Sonnenschein (Boston). We thank Dr Michael Grotzer, Dr Tarek Shalaby and Dr Ana Lauber from the University of Zurich, and Dr Matthias Kamber from the Institute of Sport Sciences of Switzerland for helpful comments and advice.

Authors' affiliations

André O von Bueren, Risheng Ma, Margret Schlumpf, Walter Lichtensteiger, Institute of Pharmacology and Toxicology, University of Zurich, Switzerland

Competing interests: None.

REFERENCES

1 **Rundell KW**, Jenkinson DM. Exercise-induced bronchospasm in the elite athlete. *Sports Med* 2002;**32**:583–600.
2 **World Anti-Doping Agency**. The 2006 Prohibited List. (http://www.wada-ama.org/rtecontent/document/2006_LIST.pdf).
3 **Martineau L**, Horan MA, Rothwell NJ, *et al*. Muscling in on salbutamol. *Lancet* 1992;**340**:1094.
4 **Palmer JB**, Shepherd GL, Cifelli AT. Muscling in on salbutamol. *Lancet* 1992;**340**:1407.
5 **Broadley KJ**, Spencer PS. Muscling in on salbutamol. *Lancet* 1993;**341**:313.
6 **Beckett AH**. Clenbuterol and sport. *Lancet* 1992;**340**:1165.
7 **Yang YT**, McElligott MA. Multiple actions of beta-adrenergic agonists on skeletal muscle and adipose tissue. *Biochem J* 1989;**261**:1–10.
8 **Clarkson PM**, Thompson HS. Drugs and sport. Research findings and limitations. *Sports Med* 1997;**24**:366–84.
9 **Martineau L**, Horan MA, Rothwell NJ, *et al*. Salbutamol, a beta 2-adrenoceptor agonist, increases skeletal muscle strength in young men. *Clin Sci (Lond)* 1992;**83**:615–21.
10 **MacLennan PA**, Edwards RH. Effects of clenbuterol and propranolol on muscle mass. Evidence that clenbuterol stimulates muscle beta-adrenoceptors to induce hypertrophy. *Biochem J* 1989;**264**:573–9.
11 **Choo JJ**, Horan MA, Little RA, *et al*. Anabolic effects of clenbuterol on skeletal muscle are mediated by beta 2-adrenoceptor activation. *Am J Physiol* 1992;**263**:E50–6.
12 **Maltin CA**, Delday MI, Hay SM, *et al*. Propranolol apparently separates the physical and compositional characteristics of muscle growth induced by clenbuterol. *Biosci Rep* 1987;**7**:51–7.
13 **Szelei J**, Jimenez J, Soto AM, *et al*. Androgen-induced inhibition of proliferation in human breast cancer MCF7 cells transfected with androgen receptor. *Endocrinology* 1997;**138**:1406–12.
14 **Korner W**, Vinggaard AM, Terouanne B, *et al*. Interlaboratory comparison of four in vitro assays for assessing androgenic and antiandrogenic activity of environmental chemicals. *Environ Health Perspect* 2004;**112**:695–702.
15 **Berthois Y**, Katzenellenbogen JA, Katzenellenbogen BS. Phenol red in tissue culture media is a weak estrogen: implications concerning the study of estrogen-responsive cells in culture. *Proc Natl Acad Sci USA* 1986;**83**:2496–500.
16 **Foster BA**, Cunha GR. Efficacy of various natural and synthetic androgens to induce ductal branching morphogenesis in the developing anterior rat prostate. *Endocrinology* 1999;**140**:318–28.

17 **Soto AM**, Sonnenschein C, Chung KL, *et al*. The E-SCREEN assay as a tool to identify estrogens: an update on estrogenic environmental pollutants. *Environ Health Perspect* 1995;**103**:113–22.
18 **Papazisis KT**, Geromichalos GD, Dimitriadis KA, *et al*. Optimization of the sulforhodamine B colorimetric assay. *J Immunol Methods* 1997;**208**:151–8.
19 **Skehan P**, Storeng R, Scudiero D, *et al*. New colorimetric cytotoxicity assay for anticancer-drug screening. *J Natl Cancer Inst* 1990;**82**:1107–12.
20 **Reeds PJ**, Hay SM, Dorwood PM, *et al*. Stimulation of muscle growth by clenbuterol: lack of effect on muscle protein biosynthesis. *Br J Nutr* 1986;**56**:249–58.
21 **Reeds PJ**, Hay SM, Dorward PM, *et al*. The effect of beta-agonists and antagonists on muscle growth and body composition of young rats (*Rattus* sp.). *Comp Biochem Physiol C* 1988;**89**:337–41.
22 **Carter WJ**, Lynch ME. Comparison of the effects of salbutamol and clenbuterol on skeletal muscle mass and carcass composition in senescent rats. *Metabolism* 1994;**43**:1119–25.
23 **Caruso JF**, Signorile JF, Perry AC, *et al*. The effects of albuterol and isokinetic exercise on the quadriceps muscle group. *Med Sci Sports Exerc* 1995;**27**:1471–6.
24 **Le Panse B**, Collomp K, Portier H, *et al*. Effects of short-term salbutamol ingestion during a Wingate test. *Int J Sports Med* 2005;**26**:518–23.
25 **Collomp K**, Le Panse B, Portier H, *et al*. Effects of acute salbutamol intake during a Wingate test. *Int J Sports Med* 2005;**26**:513–7.
26 **Le Panse B**, Arlettaz A, Portier H, *et al*. Short term salbutamol ingestion and supramaximal exercise in healthy women. *Br J Sports Med* 2006;**40**:627–31.
27 **Srichana T**, Suedee R, Muanpanarai D, *et al*. The study of in vitro–in vivo correlation: pharmacokinetics and pharmacodynamics of albuterol dry powder inhalers. *J Pharm Sci* 2005;**94**:220–30.
28 **Kindermann W**, Meyer T. Inhaled beta2 agonists and performance in competitive athletes. *Br J Sports Med* 2006;**40**(Suppl 1):i43–7.

COMMENTARY

Because of the anabolic potency of b2-agonists, the World Antidoping Agency prohibited their systemic use and added them to their doping list. This class of drug is acknowledged to induce significant changes in body composition. Chronic administration of specific beta-2 adrenergic agonists, especially clenbuterol and salmeterol, has been shown to increase skeletal muscle strength and size in several species following relatively short periods of administration. This anabolic effect on skeletal muscle was, however, only found with salbutamol after intravenous administration with implanted minipumps in model animals, possibly because of its short half-life of time of elimination. Regardless, the potential mechanism(s) by which beta-2 agonists mediates anabolic effects remain unclear. The present study therefore investigates potential androgenic activity of salbutamol in vitro and shows anabolic effects of salbutamol through androgen receptor agonistic activity. Further studies will be necessary to determine whether this mechanism could occur in vivo after salbutamol administration at considerably lower levels.

Katia Collomp
UFR STAPS, France; katia.collomp@univ-orleans.fr